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# Molecular profiling of non-genotoxic hepato-carcinogenesis using differential display reverse transcription-polymerase chain reaction (ddRT-PCR)

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**Keywords :** ddRT-PCR, non-genotoxic hepatocarcinogenesis, phenobarbital, rat, WY-14,643

## SUMMARY

The technique of differential display reverse transcription-polymerase chain reaction (ddRT-PCR) has been used to produce unique profiles of up-regulated and down-regulated gene expression in the liver of male Wistar rats following short term exposure to the non-genotoxic hepatocarcinogens, phenobarbital and WY-14,643. Animals were treated for 3 days, whereupon their livers were extracted and snap frozen. mRNA was prepared from the livers and used for ddRT-PCR. Individual bands from the differential displays were extracted and cloned. False positives were eliminated by dotblot screening and true positives then sequenced and identified.

## INTRODUCTION

Safety evaluation of new chemicals usually necessitates the examination of genotoxic and carcinogenic potential using short-term in vitro and in vivo genotoxicity assays augmented by chronic bioassay tests. The short-term assays have proved useful in the early identification of potential genotoxic carcinogens, but their value is limited by observations which suggest that approximately 60% of chemicals identified as carcinogens in life-exposure studies produce mainly negative findings in short-term genotoxicity tests (1,2). Thus, there is currently no reliable and rapid means of evaluating the carcinogenic risk of new chemicals which fall into this latter group of compounds, termed non-genotoxic (or epigenetic) carcinogens.

It is now evident that non-genotoxic carcinogens constitute a group of chemicals which are not only divergent in their interspecies toxicity, but also demonstrate different target organ selectivities and mechanisms of action (3,4). Elucidation of the molecular mechanisms underlying non-genotoxic carcinogenesis is currently underway, but the picture is still far from complete. It is anticipated that a better understanding of the early changes in genetic expression following exposure to non-genotoxic carcinogens will aid development of experimental strategies to identify cellular markers which are diagnostic for this type of toxicity.

Subtractive ddRT-PCR is a recently developed technique which facilitates the preferential amplification of gene products that demonstrate altered expression in target tissue(s) following exposure to chemical stimuli. Furthermore, using this technique, no prior knowledge of the specific genes which are up/down regulated is required. In the current study, we have undertaken to develop a specific and rapid assay for non-genotoxic carcinogens using the technique of ddRT-PCR. This has allowed us to identify characteristic

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patterns of gene regulation following administration of two different non-genotoxic carcinogens (phenobarbital and Wy-14,643) and the subsequent identification of individual gene species which are regulated by this xenobiotic treatment.

## MATERIALS AND METHODS

### Animals and treatment

Phenobarbital (BDH, Poole, UK; 100 mg/kg/day) or [4-chloro-6-(2,3-xylylidino)-2-pyrimidinylthio] acetic acid (Wy-14,643) (Campo, Emmerich; 250 mg/kg/day) was administered by gavage to groups of 3 male Wistar rats (150–200 g) on three consecutive days, whilst control animals received nothing. All animals had free access to food (rat and mouse standard diet, B&K Universal, Hull, UK) and water. The animals were killed on the fourth day, whereupon their livers were excised, sliced into 0.5 cm cubes, snap frozen in liquid nitrogen and then stored at  $-70^{\circ}\text{C}$ .

### mRNA extraction

Up to 0.25 g of each frozen liver sample was ground under liquid nitrogen using a mortar and pestle. mRNA was extracted from the ground liver using Promega's PolyAtract® System 1000 (Promega, Madison, WI, USA) according to the technical manual. The mRNA was DNase-treated (Promega, final concentration 10 U/ml) before phenol/chloroform extraction and ethanol precipitation. The mRNA was resuspended at a final concentration 500–1000 ng/ $\mu\text{l}$ .

### ddRT-PCR

This was carried out using the PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Final PCR reactions were run on a 2% Metaphor agarose (FMC, Rockland, MD, USA) gel containing ethidium bromide (Sigma, Dorset, UK) and then overstained for 30 min with SYBR Green I DNA stain (FMC, 1:10 000 dilution in TAE).

### Band extraction and cloning

Each discernible band from the differential display pattern was extracted from the gel with a scalpel and

the DNA eluted using a Genelute™ Agarose Spin Column (Supelco, Bellefonte). An aliquot of the eluted DNA (5  $\mu\text{l}$ ) was re-amplified using the original ddRT-PCR nested primers and electrophoresed on a 2% agarose gel. The re-amplified band was extracted from the gel (as above) and the eluted DNA ligated directly into the TOPO TA Cloning® vector (Invitrogen, Carlsbad) before transformation in *Escherichia coli* TOP10F' One Shot™ cells (Invitrogen).

### Stage 1 screening

Twelve transformed (white) colonies from each band were grown up for 6 h in 200  $\mu\text{l}$  LB broth containing ampicillin (Sigma, 50  $\mu\text{g}/\text{ml}$ ) and 1  $\mu\text{l}$  of this amplified by PCR reaction (as specified in ddRT-PCR technical manual). One quarter of the completed reaction was electrophoresed on a standard 2% agarose gel and one quarter on a 2% agarose gel containing HA Yellow (Hanse Analytik GmbH, Bremen, Germany, 1 U/ $\mu\text{l}$ ) to discern the different cloning products. The remainder was used to prepare duplicate dotblots on Hybond N+ (nylon) membranes (Amersham, Little Chalfont, UK). Cultures containing different cloning products were grown up and a plasmid miniprep prepared from each (Wizard Plus SV Minipreps DNA Purification System, Promega) according to the manufacturer's instructions.

### Stage II screening

The duplicate dotblots were probed with: (a) the final differential display reaction; and (b) the 'reverse-subtracted' differential display reaction. To make the 'reverse-subtracted' probe, the subtractive hybridisation step of the ddRT-PCR procedure was carried out using the original tester cDNA as a driver and the driver as a tester. Probing and visualisation were carried out using the ECL Direct Nucleic Acid Labelling and Detection System (Amersham) according to the manufacturer's instructions. Those clones which were positive for (a) but negative for (b), or showed a substantially larger positive signal with (a) compared to (b), were chosen for further analysis.

### DNA sequencing

Positive clones as identified above were sequenced on an automated ABI DNA sequencer (Applied Biosystems, Warrington, UK).

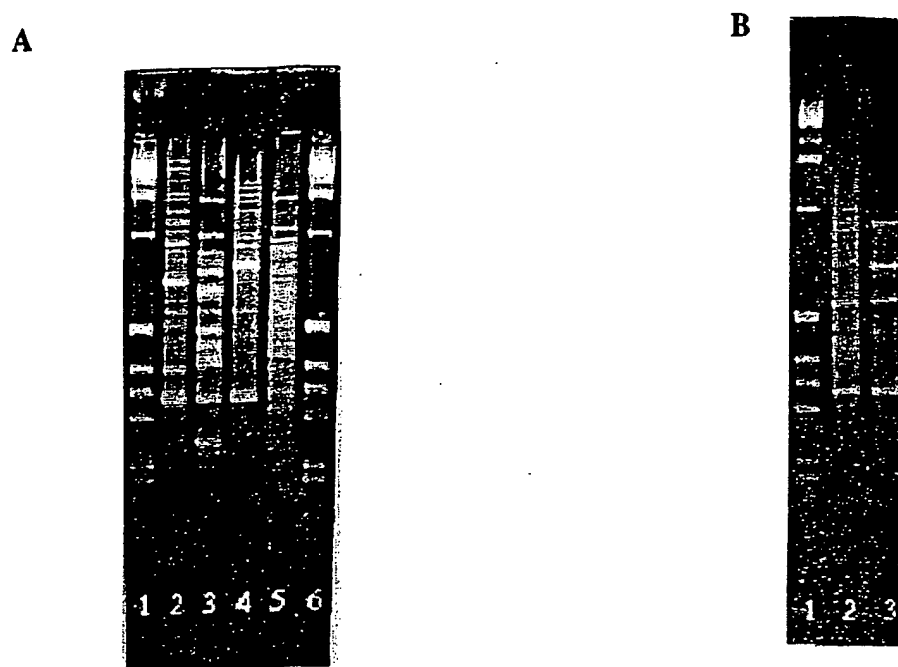


Fig. 1 : (A) Subtractive ddRT-PCR patterns obtained from rat liver following 3-day treatment with WY-14,643 or phenobarbital. Lane 1, 1 kb ladder; lane 2, genes up-regulated following Wy,14-643 treatment; lane 3, genes down-regulated following Wy,14-643 treatment; lane 4, genes up-regulated following phenobarbital treatment; lane 5, genes down-regulated following phenobarbital treatment; and lane 6, 1kb ladder. (B) Subtractive ddRT-PCR patterns obtained from rat liver showing relative changes when phenobarbital treated mRNA is subtracted from Wy-14,643-treated mRNA and vice-versa. Lane 1, 1 kb ladder; lane 2, genes showing increased expression following Wy-14,643 treatment compared to phenobarbital treatment; lane 3, genes showing increased expression following phenobarbital treatment compared to Wy-14,643 treatment. See Materials and Methods for further details.

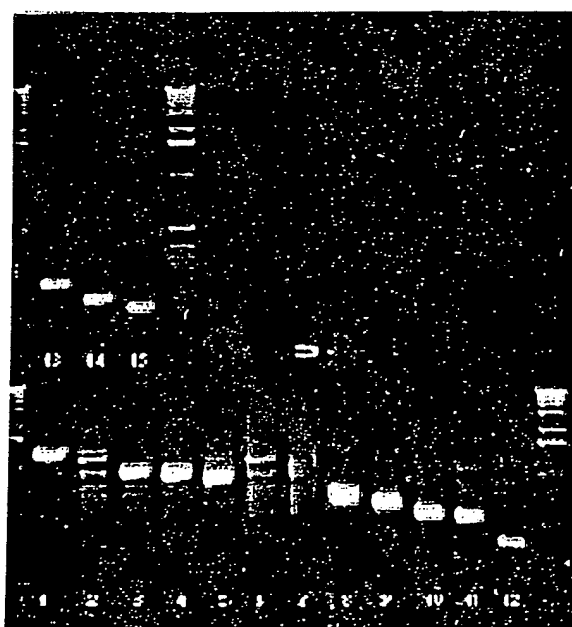


Fig. 2 : Re-amplified ddRT-PCR products which were down-regulated following phenobarbital treatment (upregulated bands were also re-amplified but gel not shown). Individual DNA bands excised from gel of ddRTR-PCR reactions were extracted, re-amplified and run on agarose gels to confirm amplification of correct band (numbered). See Materials and Methods for further details.

Table I : Rat liver genes down-regulated by phenobarbital treatment

Band number (Fig. 2)	Phenobarbital down-regulated	
(Approximate size in bp)	Highest sequence homology	FASTA-EMBL gene identification
1 (1500)	95.3%	Rat mRNA for 3-oxoacyl-CoA thiolase
2 (1200)	92.3%	Rat hemopoxin mRNA
3 (1000)	91.7%	<i>R. rattus</i> alpha-2u-globulin mRNA
7 (700)	Clone 1	77.2%
	Clone 2	94.5%
	Clone 3	91.0%
8 (650)	Clone 1	86.9%
	Clone 2	96.2%
9 (600)	Clone 1	86.9%
	Clone 2	82.0%
10 (550)	73.8%	
11 (525)	95.7%	
12 (375)	100.0%	
13 (230)	Clone 1	97.2%
	Clone 2	100.0%
	Clone 3	100.0%
14 (170)	96.0%	
15 (140)	97.3%	
Others: (300)	96.7%	
(275)	93.1%	

EST = expressed sequence tag.

Bands 4-6 were shown to be false positives by dotblot analysis and, therefore, not sequenced.

Table II : Rat liver genes up-regulated by phenobarbital treatment

Band number (Approximate size in bp)	Phenobarbital up-regulated		
	Highest sequence homology		FASTA-EMBL gene identification
5 (1300)		93.5%	Rat cytochrome P450IIB1
7 (1000)		95.1%	mRNA for rat preproalbumin Rat serum albumin mRNA
8 (950)		98.3%	NCI_CGAP_Pr1 <i>H. sapiens</i> (EST)
10 (850)		95.7%	Rat cytochrome P450IIB1
11 (800)	Clone 1	94.9%	Rat cytochrome P450IIB1
	Clone 2	75.3%	Rat cytochrome p450-L (p450IIB2)
12 (750)		93.8%	Rat TRPM-2 mRNA Rat mRNA for sulfated glycoprotein
15 (600)		92.9%	mRNA for rat preproalbumin Rat serum albumin mRNA
16 (550)	Clone 1	95.2%	Rat cytochrome P450IIB1
	Clone 2	93.6%	Rat haptoglobulin mRNA partial alpha
21 (350)		99.3%	<i>R. norvegicus</i> genes for 18S, 5.8S & 28S rRNA

EST = expressed sequence tag.

Bands 1-4, 6, 9, 13, 14 and 17-20 shown to be false positives by dotblot analysis and, therefore, not sequenced.

## Identification of differentially-regulated genes

Gene-sequences were identified using the FASTA programme (<http://www.ebi.ac.uk/htbin/fasta.py?request>) to search all EMBL databases for matching DNA sequences.

## RESULTS

Figure 1A,B shows the ddRT-PCR patterns of genes showing altered expression in rat liver following 3 day treatment with phenobarbital or Wy-14,643. Individual bands were isolated from the phenobarbital-modulated patterns (both up- and down-regulated), re-amplified (Fig. 2), cloned, screened for false positives and then identified. Those xenobiotic-modulated gene products identified to date are listed in Tables I and II.

## DISCUSSION

The advent of combinatorial chemistry has led to the synthesis of millions of new chemical compounds, many of which may be potentially useful in pharmaceutical, agricultural or industrial applications. However, whilst there are tests available for those posing a genotoxic activity, there remains no short-term assay able to identify those chemicals which may belong to the non-genotoxic group of carcinogens.

We have used an adaptation of the subtractive hybridisation method – ddRT-PCR – to produce characteristic profiles or 'fingerprints' of those genes which are up-regulated or down-regulated in male rat liver following acute exposure to test chemicals. The ddRT-PCR profiles are characteristic and unique for each of the 2 compounds studied to date.

A number of those gene species showing altered expression following phenobarbital treatment have been cloned and identified (Tables I & II). It is interesting to note the presence of CYP2B2 in the up-regulated genes. This would, of course, be expected following exposure to phenobarbital and serves as a positive control for the method. Other genes which one might normally expect to be up-regulated do not appear in the table. However, it should be noted that not

all bands seen on the differential display were extracted and re-amplified due to their being too faint or too close to other bands to accurately excise. Furthermore, it has been well documented [(5) and references therein] that a single band extracted from a differential display often represents a composite of heterogeneous products. We are currently examining new methods to: (i) improve resolution of the differential display patterns (including 2-D agarose gels); and (ii) distinguish those ddRT-PCR products which are identical in size, but different in sequence.

Our future efforts will be directed towards determining the extent of modulation of a number of the genes reported herein using semi-quantitative RT-PCR. This should reveal the extent of changes in expression of key gene products which may be involved in non-genotoxic hepatocarcinogenesis and thus help increase understanding of this process. Furthermore, it is anticipated that aligning ddRT-PCR profiles of different non-genotoxic agents found in responsive and non-responsive species may enable identification of those genes which are mechanistically relevant to the non-genotoxic hepatocarcinogenic process. Accordingly, this approach lends itself well to the identification, characterisation and sub-classification of possible different classes of non-genotoxic carcinogens.

## ACKNOWLEDGEMENT

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## REFERENCES

1. Parodi S. (1992) : Non-genotoxic factors in the carcinogenic process: problems of detection and hazard evaluation. *Toxicol. Lett.*, 64/65, 621-630.
2. Ashby J. (1992) : Prediction of non-genotoxic carcinogenesis. *Toxicol. Lett.*, 64/65, 605-612.
3. Grasso G. and Sharratt M. (1991) : Role of persistent, non-genotoxic tissue damage in rodent cancer and relevance to humans. *Annu. Rev. Pharmacol. Toxicol.*, 31, 253-287.
4. Lake B. (1995) : Mechanisms of hepatocarcinogenicity of peroxisome-proliferating drugs and chemicals. *Annu. Rev. Pharmacol. Toxicol.*, 35, 483-507.
5. Smith N.R., Li A., Aldersley M., High A.S., Markham A.E., Robinson P.A. (1997) : Rapid determination of the complexity of cDNA bands extracted from DDRT-PCR polyacrylamide gels. *Nucleic Acids Research* 25 (17). 3552-3554.

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# DRUG METABOLISM

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# Use of suppression-PCR subtractive hybridisation to identify genes that demonstrate altered expression in male rat and guinea pig livers following exposure to Wy-14,643, a peroxisome proliferator and non-genotoxic hepatocarcinogen

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## Abstract

Understanding the genetic profile of a cell at all stages of normal and carcinogenic development should provide an essential aid to developing new strategies for the prevention, early detection, diagnosis and treatment of cancers. We have attempted to identify some of the genes that may be involved in peroxisome-proliferator (PP)-induced non-genotoxic hepatocarcinogenesis using suppression PCR subtractive hybridisation (SSH). Wistar rats (male) were chosen as a representative susceptible species and Duncan–Hartley guinea pigs (male) as a resistant species to the hepatocarcinogenic effects of the PP, [4-chloro-6-(2,3-xylydino)-2-pyrimidinylthio] acetic acid (Wy-14,643). In each case, groups of four test animals were administered a single dose of Wy-14,643 (250 mg/kg per day in corn oil) by gastric intubation for 3 consecutive days. The control animals received corn oil only. On the fourth day the animals were killed and liver mRNA extracted. SSH was carried out using mRNA extracted from the rat and guinea pig livers, and used to isolate genes that were up and downregulated following Wy-14,643 treatment. These genes included some predictable (and hence positive control) species such as CYP4A1 and CYP2C11 (upregulated and downregulated in rat liver, respectively). Several genes that may be implicated in hepatocarcinogenesis have also been identified, as have some unidentified species. This work thus provides a starting point for developing a molecular profile of the early effects of a non-genotoxic carcinogen in sensitive and resistant species that could ultimately lead to a short-term assay for this type of toxicity. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Wy-14,643; Peroxisome proliferator; Non-genotoxic hepatocarcinogenesis; Suppression PCR subtractive hybridisation; RT-PCR; Rat; Guinea pig; Gene regulation; Differential gene display; Gene profiling

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## Introduction

The advent of combinatorial chemistry and computer-aided drug design has led to a recent surge in the number of chemical compounds that have potential therapeutic, agricultural and industrial applications. Although it has been suggested that the contribution of synthetic chemicals to the overall incidence of human cancer is low, there still remains an absolute requirement to evaluate all new chemicals for toxic and carcinogenic potential. The latter is one of the most problematic areas of chemical safety evaluation and is usually carried out using short-term *in vitro* and *in vivo* genotoxicity assays augmented by chronic bioassay tests. The short-term assays have proved useful in the early identification of potentially genotoxic carcinogens, but their value is limited by observations that suggest that approximately 60% of chemicals identified as carcinogens in life-exposure studies produce mainly negative findings in short-term genotoxicity tests (Lynch, 1992; Parodi, 1992). Thus, there is currently no reliable and rapid means of evaluating the carcinogenic risk of new chemicals that fall into this latter group of compounds, termed non-genotoxic (or epigenetic) carcinogens.

One approach to addressing this problem is to elucidate the molecular mechanisms by which even non-genotoxic carcinogens act. It should then be possible to identify common factors/mechanisms that can serve as early biomarkers of carcinogenic potential for new chemicals. To this end, a large number of groups have reported on the various effects of non-genotoxic compounds on various animal species (Marsman et al., 1988; Parodi et al., 1993; Cattley et al., 1994; Hayashi et al., 1994; Human and Experimental Toxicology, 1994; Anderson et al., 1996). However, the mechanistic picture is still far from complete with many of those genes involved in the carcinogenic process remaining unknown, and their identification therefore remains a key goal in elucidating the molecular mechanisms by which non-genotoxic carcinogenesis occurs.

Subtractive hybridisation (SH) and related techniques such as representational difference analysis (RDA) (Hubank and Schatz, 1994) and

differential display (DD) (Liang and Pardee, 1992) can be used to aid the isolation of genes showing altered expression in target tissues following exposure to a chemical stimulus. These techniques can also be used to identify differential gene expression in neoplastic and normal cells (Liang et al., 1992), infected and normal cells (Duguid and Dinauer, 1990), differentiated and undifferentiated cells (Sargent and Dawid, 1983; Guimaraes et al., 1995), activated and dormant cells (Gurskaya et al., 1996; Wan et al., 1996), different cell types (Hedrick et al., 1984; Davis et al., 1984) amongst others. Most importantly, using such approaches, no prior knowledge of the specific genes that are upregulated/downregulated is required.

Using a variation of SH, termed suppression-PCR subtractive hybridisation (SSH) (Diatchenko et al., 1996), we have previously reported the isolation of a number of genes showing altered expression in male rat liver following acute exposure to phenobarbital (Rockett et al., 1997). In the current work we have used the same experimental approach to isolate genes that are differentially expressed in the livers of male rats and guinea pigs following short-term (3-day) exposure to the peroxisome proliferator (PP) and non-genotoxic hepatocarcinogen, Wy-14,643. We have isolated and identified a number of gene species, some of which may be important in the induction of, or protection against, non-genotoxic hepatocarcinogenesis.

## 2. Materials and methods

### 2.1. Animals and treatment

All animal experiments were undertaken in accordance with Her Majesty's Home Office Department guidelines under the auspices of approved personal and project licences. Male Wistar rats (150–200 g) and male Duncan–Hartley guinea pigs (250–300 g) were obtained from Kingman and Bantam (Hull, UK). Upon receipt, both groups were randomly assigned into two groups of four. They were maintained on a rat, mouse or guinea pig standard diet (B&K Univer-

sal, Hull) and a daily cycle of alternating 12-h periods of dark and light. The room temperature was maintained at 19°C and a relative humidity of 55%. The animals were acclimatised to this environment for 7 days before treatment commenced. [4-chloro-6-(2,3-xylydino)-2-pyrimidinylthio] acetic acid (Wy-14,643, Campo, Emmerich; 250 mg/kg per day in corn oil) was administered by gavage to the treated groups of rats and guinea pigs on 3 consecutive days, whilst control groups received an equal volume of corn oil only. During this time, all animals had free access to food and water. The animals were killed by cervical dislocation on the fourth day, and their livers immediately excised, weighed, sliced into approximately 0.5-cm cubes, snap frozen in liquid nitrogen and stored at –70°C.

## 2.2. mRNA extraction

Approximately 0.25 g of each frozen liver sample was ground under liquid nitrogen using a mortar and pestle. Messenger RNA was extracted from the ground liver using the PolyATtract® System 1000 kit (Promega, Madison, USA) according to the technical manual provided by the manufacturers. The mRNA was DNase-treated (RQ Rnase-free Dnase, Promega, final concentration 10 U/ml) before phenol/chloroform extraction and ethanol precipitation. The mRNA was redissolved at a final concentration 500–1000 ng/µl.

## 2.3. cDNA Subtraction

This was carried out using the PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto, USA) according to the manufacturer's instructions. Subtractions were carried out with mRNAs derived from single animals. The mRNA from the remaining three animals in each group was later used for quantitative RT-PCR analysis of specific genes.

## 2.4. Band extraction and cloning

The secondary PCR reactions from the cDNA subtraction procedure were run on a 2%

Metaphor agarose gel (FMC, Rockland, USA) containing 0.5 µg/ml ethidium bromide (Sigma, Dorset, UK). One times TAE (0.04 M Tris-acetate, 0.001 M EDTA) was used to prepare the gel and as the running buffer. After running for 6–7 h at 3.75 V/cm, the gel was overstained for 30 min with SYBR Green I DNA stain (FMC, 1:10 000 dilution in 1 × TAE). Each discernible band of the differential display pattern was extracted from the gel with a scalpel and the DNA eluted using a Genelute™ agarose spin column (Supelco, Bellefonte, USA). Five microlitres of the eluted DNA was reamplified using the original nested (secondary) PCR primers supplied with the PCR-Select™ cDNA subtraction kit. The PCR products were electrophoresed on a 2% standard agarose gel (Boehringer Mannheim, East Sussex, UK) and the reamplified target bands extracted from the gel as above. The eluted DNA was immediately ligated into a TOPO TA Cloning® vector (Invitrogen, Carlsbad, USA) before transformation in *Escherichia coli* TOP10F' One Shot™ cells (Invitrogen).

## 2.5. Colony screening

### 2.5.1. Stage I

Eight transformed (white) colonies from each band were grown up for 6 h in 200 µl LB broth containing ampicillin (Sigma, 50 mg/ml). One microlitre of this was subjected to PCR using the same conditions and nested primers as described above. One tenth (2 µl) of the completed PCR reaction was electrophoresed on a 2% standard agarose gel and one tenth on a 2% standard agarose gel containing HA red (Hanse Analytik GmbH, Bremen, Germany, 1 U/ml) to discern the differentially cloned products. The remainder of the PCR reaction was used to prepare duplicate dotblots on Hybond N+ membranes (Amersham, Little Chalfont, UK).

### 2.5.2. Stage II

The duplicate dotblots were probed with (a) the final differential display reaction and (b) the 'reverse-subtracted' differential display reaction. To make the 'reverse-subtracted' probe, the subtractive hybridisation step of the differential display

RT-PCR procedure was carried out using the original tester (treated) mRNA as the driver and the original driver (control) mRNA as the tester. Probing and visualisation were carried out using the ECL direct nucleic acid labelling and detection system (Amersham, Little Chalfont, UK) according to the manufacturer's instructions. Those clones that were positive for (a) but negative for (b), or showed a substantially larger positive signal with (a) compared to (b), were selected for DNA sequence analysis.

#### 6. DNA sequencing

The remainder of the cultures (prepared in stage 1 screening) containing different cloning products (as discerned in the two screening steps) were grown up overnight in 5 ml LB broth containing ampicillin (50 mg/ml). A plasmid miniprep was prepared from each (Wizard Plus SV minipreps DNA purification system, Promega) according to the manufacturer's instructions. The cloned inserts were sequenced on an automated ABI DNA sequencer (Applied Biosystems, Warrington, UK) using the M13 forward primer (GTAAACGACGGCCAGT) or M13 reverse primer (AACAGCTATGACCATG).

#### 7. Identification of differentially regulated genes

Gene sequences thus obtained were identified using the FASTA 3.0 programme (Lipman and Pearson, 1985; Pearson and Lipman, 1988) (<http://www.ddbj.nig.ac.jp/E-mail/homology.html>) to search all EMBL databases for matching DNA sequences. Each clone sequence was submitted in the forward and reverse direction, and the one turning the highest statistical probability of match to a known sequence was noted. Sequence homologies between our submitted clone sequence and the queried database sequence were determined (by FASTA) over a region of at least 60 base pairs.

#### 8. RT-PCR analysis of selected candidate genes

cDNA sequences of the target genes were obtained from the NIH gene database (GenBank at

<http://www.ncbi.nlm.nih.gov/Web/Search/index.html>) and the computer programme GENE JOCKEY (BioSoft, Cambridge, UK) used to select primer pairs from these sequences. Where guinea pig sequences were available, rat and guinea pig sequences were aligned and primers chosen from regions of homology. If guinea pig sequences were not available, rat and human sequences were used. In cases where exact homology could not be found, the sequence from the rat was used. In the case of CD81 only, no rat or guinea pig sequences were available and so mouse and human sequences were aligned and a primer pair chosen from a region of homology. Primers (obtained from Gibco-BRL, Paisley, UK) were dissolved at a concentration of 50 pmol/μl in sterile distilled water and stored at –20°C. The primer pairs used plus other reaction parameters are shown in Table 1. mRNA was extracted (as described above) from all four treated animals and from three animals in the control group. Integrity of the eluted mRNA was confirmed on a 2% agarose gel, and the concentration and purity were measured using a Genequant II spectrophotometer (LKB, Bromma, Sweden) and then diluted to 10 ng/μl. One microlitre of this latter solution was used per RT-PCR reaction.

RT-PCR was carried out in a single tube (50 μl) reaction using the Access RT-PCR system (Promega) according to manufacturer's instructions. In the kinetic and quantitative analyses, omission of RNA was used as a control for the presence of any contaminating DNA. After obtaining a PCR signal of the correct size and optimising the reaction conditions, each PCR product was digested with between two and four separate restriction enzymes. Specific restriction patterns were thus obtained, which further confirmed the identity of the PCR products as being the original target genes. Kinetic analysis (14–32 cycles) was then performed in each case to determine the location of the mid-log phase.

For the semi-quantitative analysis of each target gene, RT-PCR reactions were carried out in triplicate for each sample to reduce the effect of intertube RT-reaction variations (Kolls et al., 1993) and pipetting errors. For each gene, a mastermix containing enough reagents for three times

Table 1  
Primer sequences and reaction conditions used in semi-quantitative RT-PCR analysis of selected genes

Transcript	Genbank accession No.	Primer sequences		Size of rat PCR product (bp)	Annealing temperature (°C) rat/guinea pig	No. of PCR cycles rat/guinea pig
		upstream	downstream			
Albumin	J00698 (rat)	TGGAGAGA-GAGC- CTTCAAAGC	CTTAG- CAAGTCTCAGCAG CA	436	60/59	15/22
Bifunctional enzyme	K03249 (rat)	GCACC- CAGTTCTTCT- CACCAGC	TGGCAATGATG- GTCCAGTAAGG	347	57/-	21/-
CYP2C11	J02657 (rat)	CCATCATGACC- CTGAGG	GAAGTCCCGAG- GATTGT	410	50/-	20/-
CYP4A1	M14972 (rat)	GATGGCTGCAC- CATGAG	GGCCTTTG- GATCTGATC	357	57/-	22/-
Catalase	M11670 (rat)	ACCAAATACTC- CAAGGCCAAAGG	GCCCTG- GTCAGTCTTG- TAATGG	450	63/-	27/-
CD81 (TAPA-1)	X59047 (mouse)	ATTTCGCTCTCTG GCTGGCTGG	GCCTGGTCATA- GAACTGCTTCA	337	57/59	23/22
Contrapsin-like protease inhibitor	RNCCP23 (rat)	GACTATGTGAG- CAATCAGAC	GTCTCTGGTTG- CAAGCT	341	50/-	20/-
Parathymosin- $\alpha$ (Zn <sup>2+</sup> binding protein)	X64053 (rat)	CGGCACCAT- GTCGGAGAAAG	TTGTGTGTTCT- GCCCCACC	382	62/-	24/-
Transferrin	D38380 (rat)	AGCTGTGT- CAACTGT- GTCCAGG	GAGGAGAGCC- GAACAGTTG- GAA	360	57/59	22/22
UDP-GT	U06273 (rat)	GGAT- GTCTGGGAAGTG	GCAGTTCAGC- TATCAGCT	495	50/-	23/-
DownUnknown-1	n/a	CGACGTTTC- CAAGGCA	TGTTGCGGCA- GAGTGGA	318	55/-	25/-
Zn $\alpha$ 2glycoprotein	D21058 (rat)	CAAATAACA- GAAGCAGTG- GAGC	GACTTCCAC- CTCCATCCAGG	433	57/-	23/-

the number of samples (seven for rat, six for guinea pig) was prepared except that mRNA was omitted, the latter being added after aliquoting 49  $\mu$ l of the mastermix into an appropriate number of tubes. Amplification of albumin (the reference gene) was carried out in separate tubes since the mid-log phase of this gene is at a much lower cycle number than the target genes due to its high abundance. All RT-PCR products were analysed on 2% agarose gels containing 0.5  $\mu$ g/ml ethidium bromide. The target gene samples were loaded on the gel first and run in at 3 V/cm for 10 min. The corresponding albumin samples were then loaded and the gel run for a further 1/2 h. In this way, all

RT-PCR products from each target gene and albumin from the corresponding samples could be run on the same gel. Gels were photographed using type 665 posi-neg film (Sigma) and quantitation of the band intensity was carried out using a dual wavelength flying spot laser scanner densitometer (Shimadzu).

### 2.9. Statistical analysis

Statistical analysis of unpaired samples was carried out using the two-tailed Student's *t*-test. Values were considered statistically significant at  $P < 0.05$  or less.

## 3. Results

### 3.1. Cloning and screening of transcripts

For both the rat and guinea pig experimental groups, cDNA subtraction was carried out in the forward (control driving tester) and reverse (tester driving control) directions to isolate both upregulated and downregulated mRNA species respectively. Using a standard primary hybridisation time of 8 h we obtained a substantial amount of non-specific products in all the final differential displays (data not shown). This background smearing was almost completely removed by reducing the primary hybridisation time to 4 h (CLONTECHniques, 1996). Fig. 1 shows the ddRT-PCR patterns of genes showing altered expression in rat and guinea pig liver following 3-day treatment with Wy-14,643. The profiles are unique for each species, and in each case the profile for the upregulated genes (control mRNA driving tester mRNA) is different to that obtained for the downregulated genes (tester mRNA driving control mRNA).

The practical outcome of the SSH method is that a series of differentially expressed genes is observed as a ladder on an agarose gel. The majority of these gene fragments fall within the 150–2000 bp range, with bands up to 5 Kbp occasionally being observed. Each band may theoretically consist of one or more products of similar size, as the gel has a maximum resolution

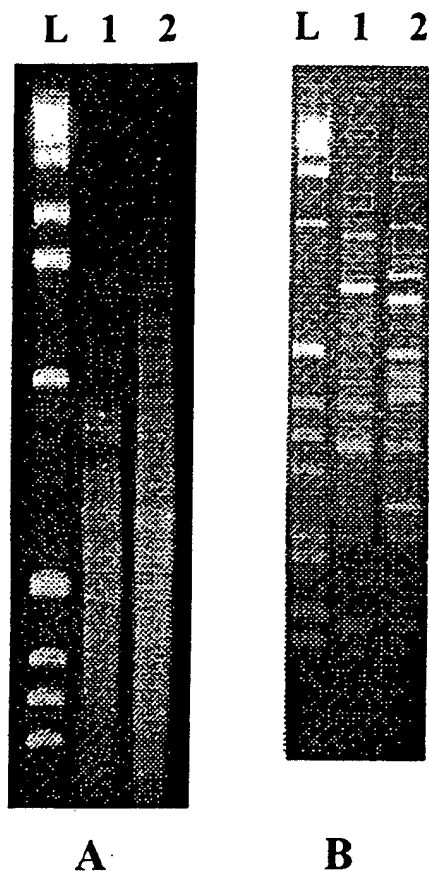


Fig. 1. Final displays of differentially expressed genes that were (1) upregulated and (2) downregulated in rat (A) and guinea pig (B) livers following 3-day treatment with Wy-14,643. mRNA extracted from control and treated livers was used to generate the differential displays using the PCR-Select cDNA subtraction kit (Clontech). Lane (L) is a 1 Kb DNA Ladder standard and 10  $\mu$ l of secondary PCR reaction were loaded in all other lanes.

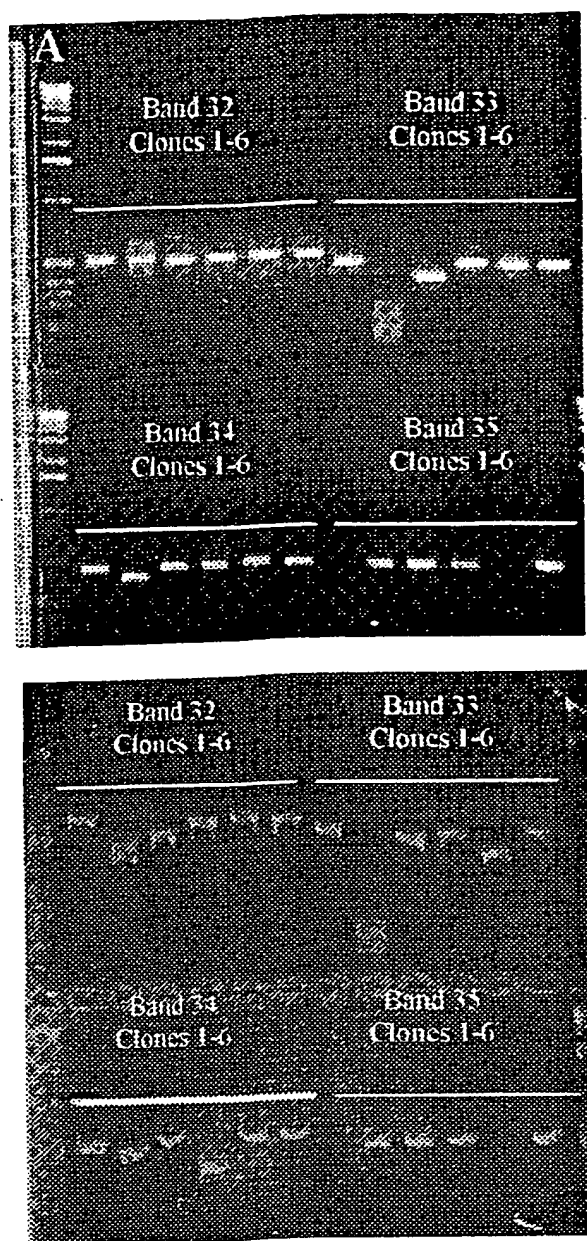


Fig. 2. Discrimination of different ddRT-PCR products having the same molecular size using HA-red. Gel (A) is a 2% standard agarose gel. Gel (B) is a 2% standard agarose gel containing 1 U/ml HA-red. Band numbers refer to the sequential bands (largest to smallest) extracted from the original display of genes upregulated in rat liver following 3-day treatment with Wy-14,643. Ten microlitres of each PCR reaction were loaded per lane.

of approximately 1.5% (3 bp per 200). In addition, there may be two or more products that are the same size, but have a different sequence.

Therefore some form of discrimination must be employed to isolate as many of these products as possible. HA-red screening (Geisinger et al., 1997) of a number of clones derived from each band provided a means to discriminate between different gene species of the same size. A typical example of such a gel is shown in Fig. 2. In total, 88 and 48 apparently different clones were obtained from the final differential expression patterns of upregulated and downregulated rat genes, respectively. Sixty nine and 89 apparently different clones were obtained from the final differential expression patterns of the upregulated and downregulated guinea pig genes, respectively.

Having identified as many different candidate gene products as possible in the screening step I, a second screening step was carried out on every clone to confirm those that represented true differentially expressed genes. This is necessary since no subtraction technique is 100% efficient. The approach we used, termed PCR-select differential screening (as recommended in Clontech's PCR-select cDNA subtraction kit protocol), utilises the forward and reverse subtractions as an aid to screening for the true differentially expressed genes (CLONTECHniques, 1997). Because these probes have already undergone subtraction, they have been enriched for differentially expressed genes and are therefore more sensitive than unsubtracted driver/tester cDNA probes for detecting true differential expression. All the clones that were isolated from each display were dotblotted and probed with the display from which they was obtained, plus the corresponding reverse-subtracted display. An example of such a blot is shown in Fig. 3. Clones corresponding to authentic differentially expressed mRNAs hybridised with the subtracted cDNA probe, but not the reverse-subtracted probe. We also included in the authentic positives, those clones that gave a substantially greater signal with the subtracted probe compared to the reverse-subtracted probe. False positives hybridised with either both probes or with neither probe. Of the original 88 upregulated and 48 downregulated rat clones selected for this screening step, 28 (32%) and 15 (31%) respectively, were found to be true positives. In the rat,

(100%) of the true positive upregulated genes (Table 2) and 11 (73%) of the true positive downregulated genes (Table 3) were non-redundant. Of the original 69 upregulated and 89 downregulated guinea pig clones selected for this screening step, 48 (70%) and 37 (42%) respectively, were found to be true positives. Thirty six (75%) of the upregulated genes (Table 4) and 33 (89%) of the downregulated genes (Table 5) were non-redundant.

## 2. Identification of clones

On sequence analysis it was found that some clones were unsequenceable in the first instance (113 forward primer) due to long polyA runs that appeared to prematurely terminate the sequencing reaction. These clones were therefore sequenced from the opposite direction using the 13 reverse primer. Those xenobiotic-modulated gene products identified to date are listed in Tables 2 and 3 (rat) and Tables 4 and 5 (guinea pig).

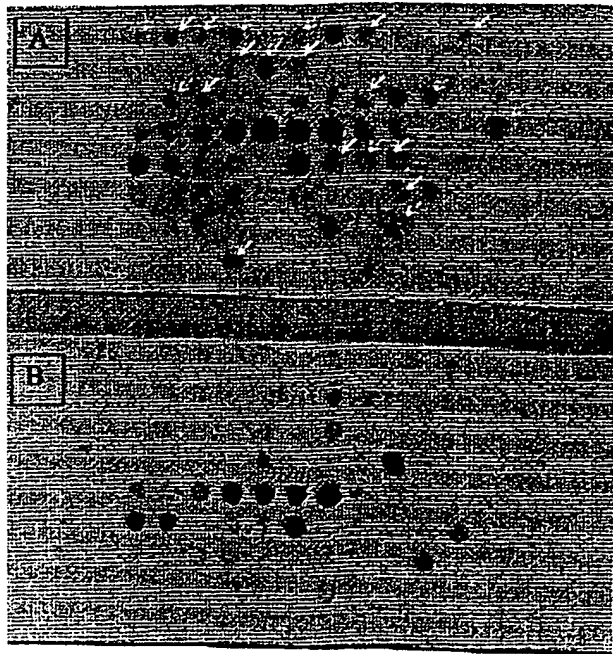


Fig. 3. Dot blots of clones of putative upregulated gene species isolated from guinea pig liver following 3-day treatment with WY-14,643. All clones identified in the stage I screening step (see methods) were blotted and probed with (A) the differential display from which they originated (control driving control) and (B) the reverse subtraction (treated driving control). Arrows indicate some of the true differentially expressed genes.

Table 2

Identification of genes that were upregulated in male rat liver following 3-day treatment with WY-14,643

FASTA-EMBL gene identification (rat unless otherwise stated)	Accession No.	Sequence homology <sup>a</sup> (%)
Carnitine octanoyl transferase	RN26033	99
NCI_CGAP_Li1 ( <i>H. sapiens</i> ) (EST <sup>b</sup> )	HS1275949	98
Peroxisomal enoyl hydratase-like protein	RN08976	98
Liver fatty acid binding protein	V01235	96
Soares mouse p3NMF19.5 <i>M. musculus</i> cDNA clone	AA038051	96
Cytochrome p450IVA1	RNCYPLA	94
Mit. 3-hydroxyl-3-methylglutaryl CoA synthase	RNHMGCOA	94
Rabgeranylgeranyl transferase component B	RNRABGERA	94
Genes for 18S, 5.8S, and 28S ribosomal RNAs	RNRRNA	94
Carnitine acetyl transferase (mouse)	MMRNACAR	92
Soares mouse NML (EST)	MM1157113	92
Bone marrow stromal fibroblast ( <i>H. sapiens</i> ) cDNA clone HBMSF2E4 (EST)	AA545726	92
7.5dpc embryo (mouse) (EST)	AA408192	92
Alpha-1-macroglobulin	RNALPH1M	91
Transferrin	RNTRANSA	91
Lecithin:cholesterol acyltransferase	RNU62803	90
Zn-α2-glycoprotein	RNZA2GA	90
Serum albumin	RNJALBM	89
Fructose-1,6-bisphosphate 1-phosphohydrolase	RNFBP	88
Soares mouse melanoma (EST) (S <sup>c</sup> )	AA124706	88
Soares mouse 3NbMS (EST) (AS <sup>c</sup> )	AA154039	88

Table 2 (Continued)

FASTA-EMBL gene identification (rat unless otherwise stated)	Accession No.	Sequence homology <sup>a</sup> (%)
17- $\beta$ -hydroxysteroid dehydrogenase	RN17BHDT2	87
Soares mouse p3NMF19.5 (EST)	AA038051	87
Peroxisomal enoyl-CoA:hydratase -3-hydroxyacyl CoA bifunctional enzyme	RNPECOA	85
Integral membrane protein, TAPA-1 (CD81) (mouse)	S45012	81
Soares mouse lymph node (EST)	MMAA88445	81
<i>H. sapiens</i> (clone zap128) mRNA	L40401	76
Lysophospholipase homologue (human)	HSU67963	76
Soares mouse lymph node (EST)	AA217044	74

<sup>a</sup> Refers to the nucleotide sequence homology between the cloned band isolated from the differential display and the corresponding gene derived from the EMBL gene sequence bank.

<sup>b</sup> EST is 'expressed sequence tag' — a gene of as yet unknown identity and function.

<sup>c</sup> Where sequence homologies were equal in both directions of the isolated band, both the sense (S) and antisense (A) identities are given.

In all cases, both the forward and reverse sequence of the target clones were analysed and the gene having the highest statistical homology noted.

### 3.3. RT-PCR analysis of selected clones

The results of a typical RT-PCR semi-quantitation experiment for transferrin in the rat is given in Fig. 4 and the results for a total of 12 selected genes in both the rat and guinea pig are shown in Table 6.

Table 3

Identification of genes that were downregulated in male rat liver following 3-day treatment with Wy-14,643

FAST-EMBL gene identification (rat unless otherwise stated)	Accession No.	Sequence homology <sup>a</sup> (%)
NCI_CGAP_Li1 ( <i>H. sapiens</i> ) (EST <sup>b</sup> )(S <sup>c</sup> )	AA484528	99
NCI_CGAP_Pr1 ( <i>H. sapiens</i> ) (EST)(AS <sup>c</sup> )	AA469320	99
UDP-glucuronosyl-transferase (UGT2B12)	RN06273	98
Complement component c3	RNC3	96
Soares mouse placenta (S)	AA023305	96
Ape (chimpanzee) 28S rRNA (AS)	PTRGMC	96
Rat CYP2C11	RNCYPM1	95
Ribosomal protein S5	RNRPS5	94
Transthyretin	RNTTHY	94
Contrapsin-like protease inhibitor	RNCCP23	89
Prostaglandin F2a (S)	RN26663	84
$\beta$ -2-microglobulin (AS)	RNB2MR	84
Apolipoprotein C-III	RNAPOA02	82
Parathymosin-alpha (zinc2 <sup>+</sup> -binding protein)	RN11ZNBP	75

<sup>a</sup> Refers to the nucleotide sequence homology between the cloned band isolated from the differential display and the corresponding gene derived from the EMBL gene sequence bank.

<sup>b</sup> EST is 'expressed sequence tag' — a gene of as yet unknown identity and function.

<sup>c</sup> Where sequence homologies were equal in both directions, both the sense (S) and antisense (A) identities are given.

## 4. Discussion

It is now apparent that all cancers arise from accumulated genetic changes within the cell. Although documenting and explaining these changes presents a formidable obstacle to understanding the different mechanisms of carcinogenesis, the experimental methodology is now available to begin attempting this difficult challenge. In order to begin the elucidation of the molecular mechanisms involved in non-genotoxic hepatocarcino-



esis, we have used SSH to identify a number of genes that are upregulated or downregulated in the rat and guinea pig livers following short term exposure to the PP, Wy-14,643. We have used the rat model to represent a species susceptible to the non-genotoxic carcinogenic effect of WY-14,643 and the guinea pig as a resistant species (Morton et al., 1984; Rodricks and Turnbull, 1987;

Lake et al., 1989; Makowska et al., 1992; Lake et al., 1993).

Gurskaya et al. (1996), who originally developed the SSH technique, cloned the products of the secondary PCR reaction and screened a small number of randomly selected colonies for differentially expressed clones using northern hybridisation. However, we decided against this approach

#### Table 4

Identification of genes that were upregulated in male guinea pig liver following 3-day treatment with WY-14,643

STA-EMBL gene identification (guinea pig unless otherwise stated)	Accession No.	Sequence homology <sup>a</sup> (%)
boxylesterase	AB010634	97
complement C3 protein (GPC3)	M34054	97
formaldehyde dehydrogenase (sheep)	U12761	92
alase (human)	X04076	89
mitochondrial aspartate aminotransferase (pig)	M11732	89
regulation factor-1-alpha (rabbit)	X62245	88
I_CGAP_Br2 <i>H. sapiens</i> cDNA clone (EST) (Similar to chick mit. phosphoenolpyruvate carboxykinase)	AA587436	87
alpha-1-antiproteinase S	M57270	83
formyltetrahydrofolate dehydrogenase (rat)	M59861	83
cytosolic protein L6 (rat)	X87107	83
res pregnant uterus Nb (EST) (mouse)	AA156847	83
mitochondrial citrate transport protein (human)	L77567	80
cytoplasmic chaperonin hTRiC5 (human)	U17104	80
alpha-1-antiproteinase F	M57271	77
heterogeneous nuclear ribonuclearprotein c1/c2 (human)	D28382	77
res parathyroid tumour (EST) (similar to human serum albumin precursor)	AA860651	76
regulate mouse kidney (EST)	AA107327	75
res parathyroid tumour NbHPA human cDNA (EST)	AA860653	74
res mouse mammary gland (EST)	AA619297	74
NA clone 15 004 (EST) (human)	H01826	74
res senescent fibroblasts (EST) (mouse)	W52190	74
proalbumin (human)	E04315	72
NA clone 73 169 (EST) (human)	T56624	72
immunin D-binding protein (human)	L10641	71
alpha-H gene (exon 8) (human)	Y11498	71
flow sorted chromosome	B05457	71
res foetal liver spleen (EST) (mouse)	AA009524	71
res foetal heart NbMH19W (EST) (mouse)	AA009421	69
res foetal heart NbHH19W <i>H. sapiens</i> cDNA clone (EST)	W94377	67
nylalanine hydroxylase (human)	U49897	67
line-5-carboxylate dehydrogenase (human)	U24266	66
methionine-S-transferase homologue (human)	U90313	65
I_CGAP_GCBI (EST) (human)	AA769294	65
protective protein (human)	M22960	64
ne 27 375 (EST) (human)	N37046	62
regulate colon ( # 937 204) <i>H. sapiens</i> cDNA clone (EST)	AA149777	62

<sup>a</sup> Refers to the nucleotide sequence homology between the cloned band isolated from the differential display and the corresponding gene derived from the EMBL gene sequence bank.

Table 5

Identification of genes that were downregulated in male guinea pig liver following 3-day treatment with WY-14,643

FASTA-EMBL gene identification (guinea pig unless otherwise stated)	Accession No.	Sequence homology <sup>a</sup> (%)
Complement C3 protein	M34054	97
Murinoglobulin	D84339	95
Alpha-1-antitrypsin	M57271	88
Elongation factor- $\alpha$ (rabbit)	X62245	89
Coupling protein G (human)	X04409	88
NCI_CGAP_Ov1 (EST <sup>b</sup> ) (human)	AA586309	87
Lecithin:cholesterol acetyl transferase (rabbit)	D13668	85
Aldolase B (human)	X00270	84
Anti-thrombin III (human)	E00116	80
Phenylalanine hydroxylase (human)	K03020	80
Inter- $\alpha$ -trypsin inhibitor (human)	D38595	79
Normalised rat muscle (EST) (S <sup>c</sup> )	AA849753	78
Normalised rat ovary (EST) (AS <sup>c</sup> )	AA801059	78
Complement factor Ba fragment (human)	X00284	77
Dihydrodiol dehydrogenase (human)	U05598	76
Spot14 gene (thyroid-inducible hepatic protein)(human)	Y08409	75
BAC clone 174p12 (human)	AC004236	75
Mitochondrial aldehyde dehydrogenase (human)	X05409	74
Preproalbumin (human)	E04315	74
NCI_CGAP_Pr9 (EST) (human) (S)	AA533142	74
Normalised rat placenta (EST) (AS)	AA851197	74
Heparin sulfate proteoglycan (human)	J04621	73
cDNA clone 33 992 (EST) (human)	R24330	73

Table 5 (Continued)

FASTA-EMBL gene identification (guinea pig unless otherwise stated)	Accession No.	Sequence homology <sup>a</sup> (%)
Retinol dehydrogenase (rat)	U33501	71
TAPA-1 integral membrane protein (CD81) (mouse)	S45012	71
Complement component c5s	M35525	70
Apolipoprotein B (pig)	L11235	69
cDNA clone 143 918 (EST) (human)	R76742	68
$\alpha$ -fibrinogen (human)	K02569	68
Soares foetal liver spleen INF (mouse)	W03726	68
Barstead bowel (EST) (mouse)	AA232049	67
UDP glucuronosyl transferase (cat)	AF0309137	66
Myeloid leukaemia cell differentiation protein (MCL-1) (human) (S)	L08246	65
STS SHGC-34 987 (human) (AS)	hu-G27984	65
Soares mouse 3NME125	AA222798	64
Stratagene mouse embryonic (EST) (S)	AA199420	64
Rad 52 (mouse)	AF004854	63

<sup>a</sup> Refers to the nucleotide sequence homology between the cloned band isolated from the differential display and the corresponding gene derived from the EMBL gene sequence bank.

<sup>b</sup> EST is 'expressed sequence tag' — a gene of as yet unknown identity and function

<sup>c</sup> Where sequence homologies were equal in both directions, both the sense (S) and antisense (A) identities are given.

for several reasons: (1) the kinetics of ligation and transformation favour the isolation of smaller PCR products, thereby producing a misrepresentation of larger gene products; (2) northern blot analysis is notoriously insensitive and is unlikely to confirm expression of rare transcripts; (3) there is no measurable end point to the screening of clones produced in this way other than to analyse every transformed colony. We used instead an alternative approach; after running out the differ-

ential display on a high-resolution agarose gel (Fig. 1) and overstaining with SYBR Green I to enhance visualisation, the composite bands were individually extracted, reamplified and cloned. However, it has been well documented that single bands from differential displays often contain a heterogeneous mixture of different products (Mathieu-Daude et al., 1996; Smith et al., 1997). This is because polyacrylamide gels cannot discriminate between DNA sequences that differ in size by less than about 0.2% (Sambrook et al., 1989). High-resolution agarose gels such as those used in this work are even less sensitive, normally only discriminating products that differ in size by at least 1.5%. The use of the HA-red screening step enables resolution of identical or nearly identical sequences based on their AT content (Wawer et al., 1995) and is sensitive down to < 1% difference. Furthermore, it is rapid, technically simple and does not require the use of radiolabels. Beisinger et al. (1997) originally demonstrated the usefulness of using HA-red to identify different products cloned from the same band of an RNA differential display experiment by simultaneously running them in normal agarose (to discriminate by size) and in normal agarose containing HA-red (to discriminate by AT content). We have found that this approach is equally useful for identifying different gene species cloned from the same band of our SSH display.

Diatchenko et al. (1996) reported that SSH is highly efficient at producing differentially expressed gene species. However, we also included a second screening step to further confirm that the clones isolated from the differential display were indeed differentially expressed. Duplicate dotblots of the candidate clones were blotted with the display from which they were originally isolated and with the 'reverse subtraction' display. To make the reverse-subtracted probe, the subtractive hybridisation step of the procedure was carried out using the original tester cDNA as a driver, and the original driver cDNA as a tester. In this way, clones that are false positives can be identified through their presence in both blots. Such false positives most commonly arise through having a very high abundance in the initial sample or unusual hybridisation properties (Li et al., 1994).

Although the SSH method itself has been shown to be efficient, and despite the screening step that we included, there is an important caveat to bear in mind — namely that it is important that all clones be considered only as 'candidates' until the actual abundance of their mRNA is quantitated in treated and control samples. Towards this end, we examined the expression of a limited number of clones using semi-quantitative RT-PCR. Albumin was used as the reference gene as we have previously found that the expression of this gene does not appear to change with the treatment regime that we used (Fig. 4, and data not shown). There are a number of interesting points to note from our results. The first is the presence of genes that serve as appropriate positive controls in the upregulated and downregulated series. For example, in the rat it can be seen that CYP4A1 expression increases 14-fold following treatment. Although CYP4A1 mRNA expression levels following WY-14,643 treatment have not been previously reported in this model, the figure compares favourably with that recorded by Bell et al. (1991), who used RNase-protection to quantitate CYP4A1 in rat liver following treatment with methylclofenapate, another PP. In addition, we also confirmed that the peroxisomal enoyl-CoA:hydratase-3-hydroxyacyl-CoA bifunctional enzyme is also upregulated 9-fold, in agreement with the findings of Chen and Crane (1992).

A number of genes were downregulated following Wy-14,643 exposure, including CYP2C11 expression. Corton et al. (1997) reported similar findings and suggested that this may in part explain why male rats exposed to Wy-14,643 and some other PPs have high serum estradiol levels, as estradiol is a substrate for CYP2C11. We have also shown that the expression of contrapsin-like protease inhibitor (CLPI) was downregulated by Wy-14,643. This has not previously been reported, and we suggest that it may be linked to a requirement for increased availability of amino acids to accommodate the hepatomegaly induced by treatment. Although little is known of the function of parathymosin- $\alpha$ , (zinc<sup>2+</sup>-binding protein) it has been shown to interact with the globular domain of histone H1, suggesting a role in histone function (Kondili et al., 1996). In contrast to the

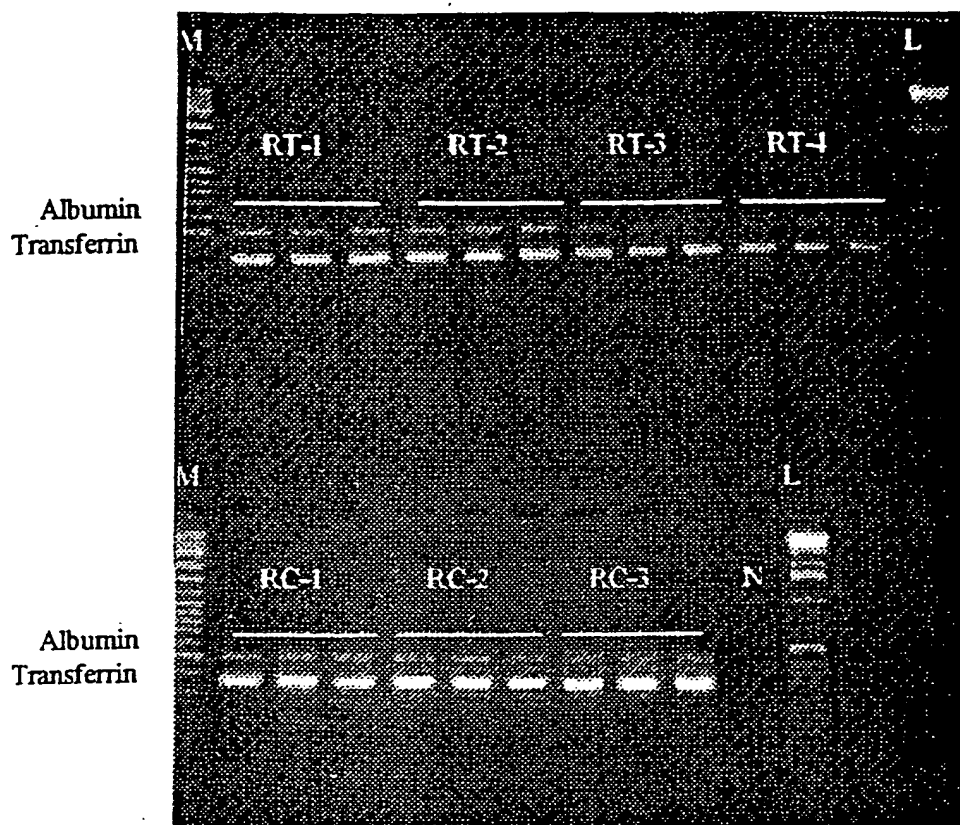


Fig. 4. Semi-quantitative RT-PCR experiment showing relative decrease in expression of transferrin in treated rat liver (RT-1 to RT-4) compared to controls (RC-1 to RC-3). An equal amount of mRNA was used in each reaction (10 ng), and each sample was quantitated in triplicate to reduce the effects of inter-tube variation. N is negative control (no mRNA). Lane M is a 100 bp ladder and lane L is a 1 Kb DNA ladder.

downregulation observed in this work, other studies have shown that parathymosin- $\alpha$  expression is elevated in breast cancer (Tsitsilonis et al., 1993, 1998), with the implication that parathymosin- $\alpha$  may somehow be involved in regulating cell proliferation by more than one mechanism. Transferrin has previously been shown to be downregulated in rat liver by hypolipidemic PPs (Hertz et al., 1996). It is therefore interesting to note that we isolated a clone identified as transferrin from the upregulated display profile. Since we confirmed by RT-PCR that transferrin is in fact downregulated in the rat (Fig. 4), we conclude that transferrin was either a false positive or was incorrectly identified. It could also be that we have isolated a close relative, splice variant or isoform of transferrin, which demonstrates a different expression profile under these experimental conditions. Further investigations are therefore

required to determine which of these possibilities are correct.

One of our most intriguing observations was that one gene, CD81, appeared to be upregulated in rat liver but downregulated in guinea pig liver following Wy-14,643 exposure. CD81 is a widely expressed cell surface protein that is involved in a large number of cellular functions, including adhesion, activation, proliferation and differentiation (reviewed by Levy et al., 1998). Since all of these functions are altered to some extent in carcinogenesis, it is perhaps an important observation that CD81 expression is differentially regulated in a resistant and sensitive species exposed to a non-genotoxic carcinogen.

Albumin and ribosomal genes appear common to all differential displays and are thus undesirable false positives. However, due to their high expression in the liver, they are difficult to re-

move. We also noted a number of gene species, particularly in the guinea pig, which were common to both upregulated and downregulated profiles. Again, the most likely reason for these having arisen is their high abundance.

A relatively large number of upregulated and downregulated genes were isolated from guinea pig liver following Wy-14,643 exposure. However, the guinea pig genome has been relatively poorly characterised and so many of the clones were identified as resembling genes or ESTs from other species. Without full-length sequence data it is difficult to ascertain the accuracy of the assigned identities and this must be borne in mind when utilising data such as this, for example, in designing effective primers for RT-PCR studies. Although the actual isolated clone sequences can be used to do this, their relatively small size often restricts the ability to design effective primers. In addition, as we observed with transferrin, using a published full-length sequence may help to identify false positives.

By comparing the expression profiles of genes showing altered expression in a PP-sensitive species (rat) with a PP-resistant species (guinea pig), it was our aim to identify genes that are mechanistically relevant to the non-genotoxic hepatocarcinogenic action of Wy-14,643. However, few of the genes that we have isolated were common to both the rat and the guinea pig. This suggests either that the molecular mechanisms of response in these two species are so different that few genes are commonly regulated in response to Wy-14,643 exposure, or that we have recovered only a small proportion of those genes that have altered expression. The latter seems the more likely scenario since it is perceived that one of the main problems of subtractive hybridisation and other differential expression technologies is the inability to consistently isolate rare gene transcripts (Bertioli et al., 1995). This is potentially problematic in that weakly expressed genes may play an important role in regulating key cellular processes, and that the majority of mRNA species are classified as

Table 6  
Semi-quantitative RT-PCR analysis of selected gene species in the rat and guinea pig<sup>a</sup>

Transcript	Putative change of expression following treatment according to dotblot		Change according to RT-PCR quantitation	
	Rat	Guinea pig	Rat	Guinea pig
Albumin	N/A	N/A	No change	No change
Alb functional enzyme	Up	N/A	Upregulated* (9 × )	N/O
YP2C11	Down	N/A	Downregulated* (Abolished)	N/D
YP4A1	Up	N/A	Upregulated* (14 × )	N/D
Alkaline phosphatase	N/A	Up	No change	N/O
D81 (TAPA-1)	Up	Down	N/O	Upregulated** (1.4 × )
Contrapsin-like protease inhibitor	Down	N/A	Downregulated** (0.5 × )	N/D
Parathymosin-α (zinc <sup>2+</sup> binding protein)	Down	N/A	Downregulated** (0.6 × )	N/D
Transferrin	Up	N/A	Downregulated* (0.5 × )	No change
DP-Glucuronosyl transferase	Down	N/A	Downregulated** (0.2 × )	N/O
Unknown-1	Down	N/A	No change (P = 0.06)	N/D
α-2-glycoprotein	Up	N/A	No change	N/O

<sup>a</sup> N/A, not applicable; N/O, not optimised; N/D, not done.

\* P < 0.0005;

\*\* P < 0.05.

'rare' in abundance (Bertioli et al., 1995). However, in their original paper describing the SSH technique, Gurskaya et al. (1996) demonstrated that SSH can enrich rare molecules between 1000- and 5000-fold in a single round of hybridisation. Unfortunately, due to high background smearing in our initial experiments (which hindered identification of single bands), we were compelled to reduce the primary hybridisation time to only 4 h — a step that theoretically is likely to reduce the number of rare sequences (CLONTECHniques, 1996). Furthermore, it has been claimed by the manufacturers that, whilst this technique can identify changes as small as 1.5-fold between the driver and tester populations, it is best suited to the isolation of genes that show a greater than 5-fold increase (CLONTECHniques, 1996). In addition, where tester and driver contain genes with large and small differences in abundance, the SSH method will be biased towards identifying those genes with the large differences (CLONTECHniques, 1996). Thus, it is most probable that we have not isolated all of the more rarely expressed transcripts and those demonstrating small changes in expression.

One problem that remains is identifying the function of genes isolated in SSH experiments as described herein, some of which may be crucial to the process of carcinogenesis, and are, to date, unidentified. However, we have provided evidence herein that SSH can be used to begin the process of characterising the extent and importance of altered gene expression in response to a chemical stimulus. The developments of this approach should include characterisation of temporal and dose responses, and functional analysis studies including knockout mice. In combination, such studies should make a significant contribution to our understanding of the molecular mechanisms of action and physiological relevance of gene regulation in non-genotoxic hepatocarcinogenesis. It should then be possible to ascertain whether differentially expressed genes are causally or casually related to the chemical-induced toxicity, and therefore a substantial mechanistic advance.

It is clear that there are also broader applications for this experimental approach that go beyond understanding the molecular mechanisms of

peroxisome-proliferator induced non-genotoxic hepatocarcinogenesis in rodents. The potential medical and therapeutic benefits of elucidating the molecular changes that occur in any given cell in progressing from the normal to the carcinogenic (or other diseased, abnormal or developmental) state are very substantial. Notwithstanding the lack of complete functional identification of altered gene expression, such gene profiling studies described herein essentially provides a 'fingerprint' of each stage of carcinogenesis, and should help in the elucidation of specific and sensitive biomarkers for different types of cancer. Amongst other benefits, such fingerprints and biomarkers could help uncover differences in histologically identical cancers, and provide diagnostic tests for the earliest stages of neoplasia. In addition, the genes identified by this approach may be incorporated into gene-chip DNA-arrays, thus providing a standard genetic fingerprint for a particular toxin treatment in a particular species. Interrogation of these gene arrays for an unknown compound that has a similar pattern to the known reference chemical would then provide evidence that the unknown may have a toxicity profile similar to the 'standard' fingerprint, thereby serving as a mechanistically relevant platform for further detailed investigations.

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### References

- Anderson, N.L., Esquer-Blasco, R., Richardson, F., Foxworthy, P., Eacho, P., 1996. The effects of peroxisome proliferators on protein abundances in mouse liver. *Toxicol. Appl. Pharmacol.* 137, 75–89.
- Ashby, J., 1992. Prediction of non-genotoxic carcinogenesis. *Toxicol. Lett.* 64–65, 605–612.
- Bell, D.R., Bars, R.G., Gibson, G.G., Elcombe, C.R., 1991. Localisation and differential induction of cytochrome P450IVA and acyl coA oxidase in rat liver. *Biochem. J.* 275, 247–252.
- Bertioli, D.J., Schlichter, U.H.A., Adams, M.J., Burrows, P.R., Steinbiss, H.-H., Antoniw, J.F., 1995. An analysis of

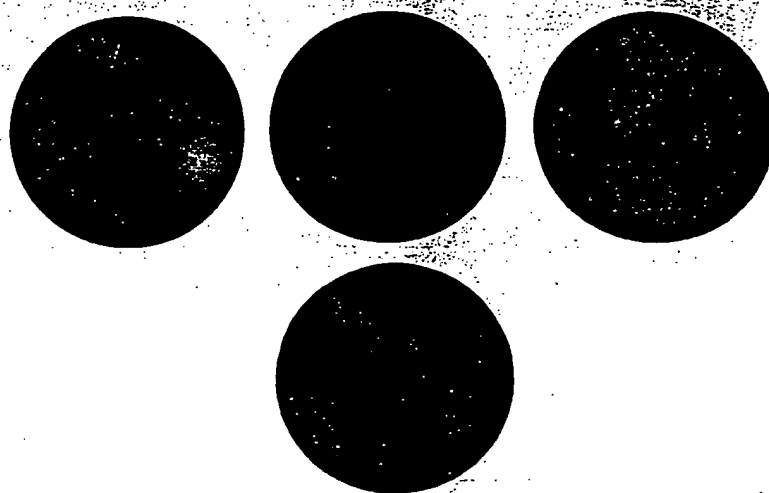
- differential display shows a strong bias towards high copy number mRNAs. *Nucleic Acid Res.* 23 (21), 4520–4523.
- Cattley, R.C., Kato, M., Popp, J.A., Teets, V.J., Voss, K.S., 1994. Initiator-specific promotion of hepatocarcinogenesis by Wy-14,643 and clofibrate. *Carcinogenesis* 15 (8), 1763–1766.
- Chen, N., Crane, D.I., 1992. Induction of the major integral membrane protein of mouse liver peroxisomes by peroxisome proliferators. *Biochem J.* 283, 605–610.
- CLONTECHniques, 1996. Technical Tips: Clontech PCR-Select cDNA Subtraction, October 25, application notes.
- CLONTECHniques, 1997. PCR-Select Differential Screening Kit — The Next Step After Clontech PCR-Select cDNA Subtraction. XII(2), 18–19, application notes.
- Corton, J.C., Bocos, C., Moreno, E.S., Merrit, A., Cattley, R.C., Gustaffson, J.A., 1997. Peroxisome proliferators alter the expression of estrogen-metabolising enzymes. *Biochimie* 79, 151–162.
- Davis, M., Cohen, D.I., Nielson, E.A., Steinmetz, M., Paul, W.E., Hood, L., 1984. Cell-type-specific cDNA probes and the murine I region: the localisation and orientation of Ad/a. *Proc. Natl. Acad. Sci. USA* 81, 2194–2198.
- Diatchenko, L., Lau, Y.-F.C., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, K., Gurskaya, N., Sverdlov, E.D., Siebert, P.D., 1996. Suppression subtractive hybridisation: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* 93, 6025–6030.
- Duguid, J., Dinauer, M., 1990. Library subtraction of in vitro cDNA libraries to identify differentially expressed genes in scrapie infection. *Nucleic Acid Res.* 18 (9), 2789–2792.
- Geisinger, A., Rodriguez, R., Romero, V., Wettstein, R., 1997. A simple method for screening cDNAs arising from the cloning of RNA differential display bands. Elsevier trends journals technical tips online, <http://tto.trends.com>, document number T01110
- Guimaraes, M.J., Lee, F., Zlotnik, A., McClanahan, T., 1995. Differential display by PCR: novel findings and applications. *Nucleic Acid Res.* 23 (10), 1832–1833.
- Gurskaya, N.G., Diatchenko, L., Chenchik, P.D., Siebert, P.D., Khaspekov, G.L., Lukyanov, K.A., Vagner, L.L., Ermolaeva, O.D., Lukyanov, S.A., Sverdlov, E.D., 1996. Equalising cDNA subtraction based on selective suppression of polymerase chain reaction: cloning of Jurkat cell transcripts induced by phytohemagglutinin and phorbol 12-myristate 13-acetate. *Anal. Biochem.* 240, 90–97.
- Hayashi, F., Tamura, H., Yamada, J., Kasai, H., Suga, T., 1994. Characteristics of the hepatocarcinogenesis caused by dehydroepiandrosterone, a peroxisome proliferator, in male F-344 rats. *Carcinogenesis* 15 (19), 2215–2219.
- Hedrick, S.M., Cohen, D.I., Nielsen, E.A., Davis, M.M., 1984. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature* 308 (8), 149–153.
- Hertz, R., Seckbach, M., Zakim, M.M., Bar-Tana, J., 1996. Transcriptional suppression of the transferin gene by hypolipidemic peroxisome proliferators. *J. Biol. Chem.* 271 (1), 218–224.
- Hubank, M., Schatz, D.G., 1994. Identifying differences in mRNA expression by representational difference analysis. *Nucleic Acid Res.* 22 (25), 5640–5648.
- Human and Experimental Toxicology, 1994. *Hum. Exp. Toxicol.* 13 (Suppl. 2) (entire issue).
- Kolls, J., Dsininger, P., Cohen, C., Larson, J., 1993. cDNA equalisation for reverse transcription-polymerase chain reaction quantitation. *Anal. Biochem.* 208, 264–269.
- Kondili, K., Tsolas, O., Papamarcaki, T., 1996. Selective interaction between parathymosin and histone H1. *Eur. J. Biochem.* 242 (1), 67–74.
- Lake, B.G., Evans, J.G., Gray, T.J.B., Korosi, S.A., North, C.J., 1989. Comparative studies of nafenopin-induced hepatic peroxisome proliferation in the rat, Syrian hamster, guinea pig and marmoset. *Toxicol. Appl. Pharmacol.* 99, 148–160.
- Lake, B.G., Evans, J.G., Cunningham, M.E., Price, R.J., 1993. Comparison of the hepatic effects of Wy-14,643 on peroxisome proliferation and cell replication in the rat and Syrian hamster. *Environ. Health Perspect.* 101 (S5), 241–248.
- Levy, S., Todd, S.C., Maecker, H.T., 1998. CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu. Rev. Immunol.* 16, 89–109.
- Li, W.B., Gruber, C.E., Lin, J.J., D'Alessio, J.M., Jessee, J.A., 1994. The isolation of differentially expressed genes in fibroblast growth factor stimulated BC3H1 cells by subtractive hybridization. *BioTechniques* 16, 722–729.
- Liang, P., Pardee, A.B., 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257 (5072), 967–971.
- Liang, P., Averboukh, L., Keyomarsi, K., Sager, R., Pardee, A.B., 1992. Differential display and cloning of messenger RNAs from human breast cancer versus mammary epithelium. *Cancer Res.* 52, 6966–6968.
- Lipman, D.J., Pearson, W.R., 1985. Rapid and sensitive protein similarity searches. *Science* 227, 1435–1441.
- Makowska, J.M., Gibson, G.G., Bonner, F.W., 1992. Species differences in ciprofibrate induction of hepatic cytochrome P450IVA1 and peroxisome proliferation. *J. Biochem. Toxicol.* 7, 183–191.
- Marsman, D.S., Cattley, R.C., Conway, J.G., Popp, J.A., 1988. Relationship of hepatic peroxisome proliferation and replicative DNA synthesis to the hepatocarcinogenicity of the peroxisome proliferators di-(2-ethylhexyl)phthalate and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) in rats. *Cancer Res.* 48, 6739–6744.
- Mathieu-Daude, F., Cheng, R., Welsh, J., McClelland, M., 1996. Screening of differentially amplified cDNA products from RNA arbitrarily primed PCR fingerprints using single strand conformation polymorphism (SSCP) gels. *Nucleic Acid Res.* 24 (8), 1504–1507.
- Orton, T.C., Adam, H.K., Bentley, M., Holloway, B., Tucker, M.J., 1984. Clobuzarit: species differences in the morphological and biochemical response of the liver following chronic administration. *Toxicol. Appl. Pharmacol.* 73, 138–151.

- Parodi, S., 1992. Non-genotoxic factors in the carcinogenic process: problems of detection and hazard evaluation. *Toxicol. Lett.* 64–65, 621–630.
- Pearson, W.R., Lipman, D.J., 1988. Imported tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* 85, 2444–2448.
- Rockett, J.C., Esdaile, D.J., Gibson, G.G., 1997. Molecular profiling of non-genotoxic hepatocarcinogenesis using differential display reverse transcription-polymerase chain reaction (ddRT-PCR). *Eur. J. Drug. Metab. Pharmacokinet* 22 (4), 329–333.
- Rodricks, J.V., Turnbull, D., 1987. Inter-species differences in peroxisomes and peroxisome proliferation. *Toxicol. Ind. Health* 3, 197–212.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. In: Ford, N., Nolan, C., Ferguson, M. (Eds.), *Molecular Cloning — A Laboratory Manual*, second ed. Cold Spring Harbor Laboratory Press, New York.
- Sargent, T., Dawid, I., 1983. Differential gene expression in the gastrula of *Xenopus laevis*. *Science* 222, 135–139.
- Smith, N.R., Li, A., Aldersley, M., High, A.s., Markham, A.F., Robinson, P.A., 1997. Rapid determination of the complexity of cDNA bands extracted from DDRT-PCR polyacrylamide gels. *Nucleic Acid Res.* 25 (17), 3552–3554.
- Tsitsilonis, O.E., Stiakakis, J., Koutselinis, A., Gogas, J., Markopoulos, C., Yialouris, P., Bekris, S., Panoussopoulos, D., Kiortsis, V., Voelter, W., Haritos, A.A., 1993. Expression of alpha-thymosins in human tissues in normal and abnormal growth. *Proc. Natl. Acad. Sci. USA* 90 (20), 9504–9507.
- Tsitsilonis, O.E., Bekris, E., Voutsas, I.F., Baxevanis, C.N., Markopoulos, C., Papadopoulou, S.A., Kontzoglou, K., Stoeva, S., Gogas, J., Voelter, W., Papamichail, M., 1998. The prognostic value of alpha-thymosins in breast cancer. *Anticancer Res.* 18 (3A), 1501–1508.
- Wan, J.S., Sharp, S.J., Poirier, G.M.-C., Wagaman, P.C., Chambers, J., Pyati, J., Hom, Y.-L., Galindo, J.E., Huvar, A., Peterson, P.A., Jackson, M.R., Erlander, M.G., 1996. Cloning differentially expressed mRNAs. *Nat. Biotechnol.* 14, 1685–1691.
- Wawer, C., Ruggeberg, H., Meyer, G., Muyzer, G., 1995. A simple and rapid electrophoresis method to detect sequence variation in PCR-amplified DNA fragments. *Nucleic Acid Res.* 23 (23), 4928–4929.



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